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Inhibited enzyme activities in soil macroaggregates contribute to enhanced soil carbon sequestration under afforestation in central China

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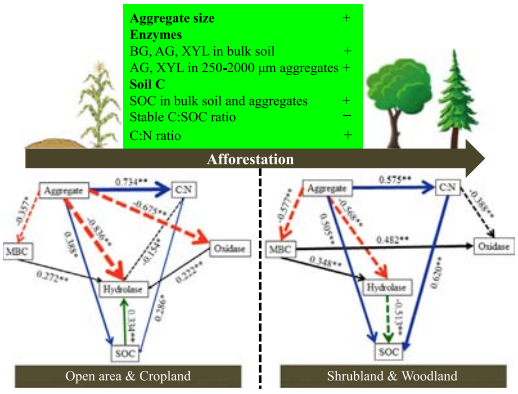
HIGHLIGHTS

- Hydrolases correlated negatively with SOC in aggregates under afforestation.
- Increased aggregate size negatively affected hydrolases in afforested soils.
- Inhibited enzymes in macroaggregates favored SOC sequestration under afforestation.

GRAPHICAL ABSTRACT



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ABSTRACT

The fate of soil organic carbon (SOC) sequestered by afforestation is crucial for the mitigation of the anthropogenic climate change but remains largely unclear. This lack of knowledge is particularly true for SOC turnover driven by enzyme activity. Here we measured hydrolase (including β-glucosidase, α-glucosidase, cellobiohydrolase and xylanase) and oxidase (including polyphenol oxidase and peroxidase) activities in soil aggregates following 30-year afforestation in central China. We also analyzed the relationships of enzyme activities with SOC concentrations, soil C:nitrogen (N) ratios and $\delta^{13}C$ values of soil organic pool (removing any carbonates by acid hydrolysis) and stable pool (NaOCl–resistant). Afforestation significantly enhanced soil β-glucosidase, α-glucosidase and xylanase activities in bulk soil, as well as SOC concentrations in bulk soil and all aggregate fractions compared to those in the open area and cropland. In particular, the woodland increased SOC concentration in >2000 μm macroaggregates by 4.2- and 3.2-fold, compared to the open area and cropland, respectively. Soil hydrolase activities were generally lower but SOC concentrations were higher in >2000 μm macroaggregates compared with those in other aggregate fractions following afforestation. Hydrolase activities were negatively correlated with SOC and C:N ratios in soil aggregate fractions following afforestation. Results of structural equation modeling indicated that the increasingly inhibited hydrolase activities with increasing soil aggregate size indirectly promoted SOC sequestration following afforestation. In addition, both hydrolase and oxidase were positively correlated with $\delta^{13}C$ values in the stable pool of the afforested soils, confirming the essential role of

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enzymes for SOC turnover in soil aggregates following afforestation. Overall, our results highlight the importance of unevenly distributed enzyme activities among soil aggregates in regulating SOC sequestration following afforestation.

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1. Introduction

Soils store up to 2500 Pg carbon (C) to 1 m depth globally, more than three times the amount that is stored in the atmosphere (Lal, 2008). It has been postulated that a 5–15% increase in soil organic C (SOC) content to 2 m depth could potentially reduce atmospheric carbon dioxide (CO_2) by 16–30% (Baldock, 2007; Kell, 2011). These ideas have led to strong interest in practices that promote C sequestration in soils, including afforestation (Wang et al., 2016; Liu et al., 2017; Nath et al., 2018). Afforested soils have been shown as a sink for atmospheric CO_2 at the ecosystem, regional and global scales (Six et al., 2002a; Hernandez-Ramirez et al., 2011; Wang et al., 2016). However, the subsequent turnover of sequestered SOC due to processes such as decomposition has received less attention (Chomel et al., 2014); through decomposition, afforested soils can also act as a potential source of atmospheric CO_2. Thus, a better understanding of the underlying mechanisms of SOC dynamics is crucial for predicting the potential of afforestation to sequester C and mitigate the anthropogenic climate change.

The SOC decomposition process is predominantly catalyzed by a sequence of enzymes, including hydrolase and oxidase (Tabatabai and Dick, 2002; Sinsabaugh, 2010). Soil enzyme activities are sensitive to land-use changes and have been commonly used as indicators of C availability and soil quality (Trasar-Cepeda et al., 2008; Burns et al., 2013). However, despite extensive researches, the evidence for the impact of afforestation on soil enzymes remains uncertain (Trasar-Cepeda et al., 2008; Wallenius et al., 2011; Singh et al., 2012). This uncertainty can be attributed to changes in SOC quantity and quality (Six et al., 2002a; Smal and Olszewska, 2008), as well as changes in microbial biomass abundance and community structure following afforestation (Trasar-Cepeda et al., 2008; Zhang et al., 2016). In addition, soil enzyme activities are unevenly distributed in different physical locations within the soil structure (Ling et al., 2014; Bach and Hofmockel, 2016; Cenini et al., 2016). The various characteristics of soil aggregate fractions can induce differences in their responses to afforestation (Fansler et al., 2005) and hence contribute to contradictory responses of bulk soil enzyme activities to afforestation. The determination of enzyme activities in the bulk soil thus may not be sensitive enough to detect the response of SOC turnover to afforestation. Although some studies have evaluated changes in soil enzyme activities in the bulk soil under afforestation (Wallenius et al., 2011; Singh et al., 2012), how soil enzyme activities in aggregate fractions respond to afforestation remains largely unclear.

The decomposition of SOC via enzymes is tightly associated with the protection mechanisms of SOC compounds (Dungait et al., 2012; Cenini et al., 2016). SOC protected by different mechanisms, including by soil aggregation, mineral sorption and intrinsic molecular recalcitrance (Six et al., 2002a; Mikutta et al., 2006; Dungait et al., 2012; O'Brien and Jastrow, 2013), varies in their decomposition potentials and intrinsic decay rates (Bird et al., 2002; Mikutta et al., 2006). Several studies have indicated that the decomposition process is regulated more by the physical accessibility of SOC to enzymes than by the biochemical recalcitrance of SOC (Dungait et al., 2012; O'Brien and Jastrow, 2013). Soil chemical fractionation after the physical separation of SOC pool has been considered as an effective approach for evaluating the relative contributions of these different mechanisms, but has rarely been tested empirically (Dungait et al., 2012). Additionally, analysis of the natural abundance of ^{13}C offers an excellent approach to detect small shifts in SOC dynamics when land use change is accompanied by a shift between C_3 and C_4 plant species (e.g., Del Galdo et al., 2003; Cheng et al., 2013). SOC dynamics and their feedbacks following afforestation can thus be further elucidated if isotopic analyses are supplemented to quantify the overall SOC transformation rates (Guillaume et al., 2015).

In the Danjiangkou Reservoir area, a crucial water source for the Middle Route of the South-to-North Water Transfer Project in China, large areas of uncultivated land were converted to shrubland and woodland approximately 30-year ago to reduce soil erosion, water pollution and soil C depletion (Zhu et al., 2010). Our previous studies have reported that afforestation significantly increased SOC contents in bulk soil primarily due to increase in litter input and decrease in decay rate (Cheng et al., 2013; Deng et al., 2016). In this study, we measured the C-degrading enzyme activities (hydrolase and oxidase), C concentrations and $\delta^{13}C$ values of soil organic pool (removing any carbonates by acid hydrolysis) and stable (NaOCl–resistant) pool in soil aggregates across a land use change gradient (open area (uncultivated area), cropland, shrubland and woodland). The objective of this study is to reveal how the distribution of C-hydrolyzing enzyme activities in soil aggregate fractions affects the turnover and sequestration of SOC following afforestation. We hypothesized that 1) soil enzyme activities in bulk soil and aggregate fractions would increase following afforestation, reflecting the increases in organic substrates due to increasing litter inputs (Trasar-Cepeda et al., 2008); 2) soil enzymes would be lower and SOC concentration would be higher in macroaggregates compared to those in finer soil aggregate particles, because of the physical isolation of SOC to enzymes in macroaggregates (Dungait et al., 2012).

2. Materials and methods

2.1. Site description and experimental design

This study was conducted at the Wulongchi Experimental Station (32°45′N, 111°13′E) in the Danjiangkou Reservoir region (Cheng et al., 2013; Wu et al., 2016). Altitude at the station is approximately 280–400 m above sea level. This region has a subtropical monsoon climate, with marked annual variations in both precipitation and temperature. Mean annual precipitation is 749.3 mm, with 70–80% falls between April and October. Mean annual temperature is 15.7 °C, which ranges from approximately 4.2 °C in January to 27.3 °C in July. The soil is a loamy clay with 11% sand, 41% silt and 48% clay (Zhu et al., 2010), and is classified as Haplic luvisols according to Food and Agriculture Organization (1993). The soils are formed primarily from limestone and red sandstone, which have a pH range of 8.17–8.53 with strong alkali carbonate reaction (Zhang et al., 2016). Human activities, such as tillage and deforestation have led to soil erosion, water pollution and soil C and N depletion in this region (Zhu et al., 2010). In 1980s, large areas of uncultivated land (open area) around the reservoir were converted to woodland (*Platycladus orientalis* (*Linn.*) *Franco*) and shrubland plantations (*Sophora davidii* (*Franch.*) *Skeels* and *Amorpha fruticosa Linn.*) (Zhu et al., 2010). Maize and wheat were grown in rotation in cropland by conventional agricultural practices, including plowing to a 0.4 m depth and mineral fertilizations. The aboveground biomass of maize and wheat were removed through harvesting.

In this experiment, four land use types (woodland, shrubland, cropland and open area) were replicated four times (four blocks) using a randomized complete block design. Each block was approximately 3 ha (600 m × 50 m). The distance between two adjacent blocks was approximately 100 m. Comprehensive surveys of soil and vegetation were conducted in April 2017 to ensure the comparability (e.g., similar topographies and soil types) of the soil sampling plots among the four land types.

2.2. Field sample collection and soil aggregate fractionation

In April 2017, five sub-plots (2 m × 2 m) were randomly selected for each land use type in each block. Within each sub-plots, three soil cores (0–10 cm depth, 5 cm diameter) were collected at random after removing the litter. Then 15 soil cores from five sub-plots were combined to yield one composite sample for each plot (i.e., each land use type in each block). A portion of each soil sample was stored at 4 °C for soil aggregate fractionation and the measurement of enzyme activities. Another portion of soil samples was air-dried for the determination of abiotic properties.

Soil aggregate fractionation procedure was carried out by a wet-sieving method for microbiological analysis as described by Smith et al. (2014). The size fractions collected were >2000, 250–2000, 53–250 and <53 μm (silt and clay particle-size fraction). Briefly, field-moist soils (100 g equivalent dry weight) were submerged for 5 min in de-ionized water on top of the 2000 μm sieve. Soils were sieved under-water by gently moving the sieve 3 cm vertically with 50 repetitions over a period of 2 min. The floating organic material (>2000 μm) was aspirated away. Material remaining on the sieve was transferred to an aluminum pan. Soils that passed through a particular sieve were then transferred to the next (finer) sieve and a similar process was repeated. Due to the <53 μm particles being in solution at the end of the wet-sieving process, the slurry was centrifuged at 2500 rpm for 2 min, and then decanted.

After weighting the moist fractions, aliquots (5 g) of each fraction were taken to determine moisture content. A subsample of each fraction was freeze-dried for C, N and isotopic analyses. The remaining moist fractions were stored at 4 °C for enzyme analyses within 3 d. One complete fractionation procedure took about 3 h.

2.3. Soil microbial biomass C and soil enzyme analyses

The moist samples of bulk soil and all aggregate fractions were analyzed for both microbial biomass C (MBC) and enzyme activities (Fansler et al., 2005; Marx et al., 2005; Smith et al., 2014). MBC was determined by a fumigation-extraction method (Joergensen, 1996; Smith et al., 2014). Soil hydrolase activities, including β-glucosidase (BG), α-glucosidase (AG), cellobiohydrolase (CB) and xylanase (XYL), were measured by a fluorimetric microplate assay (Marx et al., 2001). Soil enzyme activities were measured as the release rates of 4-methylumbelliferyl (MUB) from the MUB-labeled substrates (Marx et al., 2001). Briefly, soil suspension was prepared by vigorously mixing 1.00 g of soil or soil aggregate fractions with 100 mL of de-ionized water using a magnetic stir plate for 15 min. Eight aliquots (50 μL each) of soil suspension was included into a 96-well flat-black microplate using an eight-channel micropipette. Then each aliquot of soil suspension was mixed with 50 μL of acetate buffer (100 mM L^{-1}, pH 5.5) and 100 μL of substrate solution (10 μmol L^{-1}). Microplates were then incubated for 3.0 h at 30 °C. The released MUB were measured using a fluorimetric plate reader (Tecan Infinite 200 PRO, TECAN Group Ltd., Mannedorf, Switzerland) with excitation of 360 nm and emission of 450 nm. Soil enzyme activities were expressed as nmol product g^{-1} dry fraction h^{-1}.

Soil oxidase, including polyphenol oxidase (PPO) and peroxidase (PO) were measured by colorimetrical method using ι-dihydroxyphenylalanine (ι-DOPA) as substrate (Deforest, 2009). Similar to the fluorometric enzymes, 50 μL of soil suspension, 100 μL of acetate buffer (100 mmol L^{-1}, pH 5.5) and 50 μL of a 5 mmol L^{-1} ι-DOPA solution was included into a 96-well transparent microplate. Soil PO activity required the addition of 10 mL of 0.3% H_2O_2. Microplates were covered and incubated in the dark at 20 °C for 20 h. Absorbance at 450 nm was measured using a microplate spectrophotometer (Tecan Infinite 200 PRO, TECAN Group Ltd., Mannedorf, Switzerland). Oxidase activities were expressed as μmol product g^{-1} dry fraction h^{-1} (Deforest, 2009). Over 90% of soil hydrolytic enzymes and 80% of oxidase in aggregates were recovered in the present study (Table S1).

2.4. Fractionation of stable organic matter

Soil stable C (SC) and N (SN) concentration were measured by an oxidation method using NaOCl (Mikutta et al., 2006). In brief, 5 g of freeze-dried bulk soil or aggregate fraction was reacted with 50 mL of 6 wt% NaOCl (pH = 8.0; adjusted using 32% HCl). The mixture was shaken and oxidized for 6 h at 25 °C. Then another two same oxidation cycles were repeated and the mixtures were centrifuged at 2574 *g* for 5 min. After decanting the supernatant, the residues were treated with 0.5 M HCl for 24 h at room temperature to remove any carbonates in alkaline soils in this study (Harris et al., 2001). Then the residues were washed using 50 mL 1 M NaCl for two times and shaken overnight using deionized water. The residues were dialyzed (12–14,000 Da) and when the electrical conductivity was <40 μS cm^{-1}, the residues were freeze-dried. The organic C and N resistant to NaOCl treatment were SC and SN.

2.5. Carbon, nitrogen and isotopic analysis

C and N concentrations as well as $\delta^{13}C$ values of NaOCl treated and untreated samples (in bulk soil and aggregate fractions) were measured using a dry combustion method (Harris et al., 2001). Before analysis, bulk and aggregate soil samples untreated with NaOCl were also washed with 0.5 M HCl to remove carbonates (Harris et al., 2001). The C and N concentrations and $\delta^{13}C$ values were measured using an isotope ratio mass spectrometer (Thermo Finnigen, Delta-Plus, Flash, EA, 1112 Series, USA) (Harris et al., 2001). The differences between NaOCl untreated and treated organic C and N were defined as relatively labile organic C (LOC) and N (LON), respectively. The carbon isotope ratios of the samples were expressed using the following equation:

$$\delta 13C = \left[\left(\frac{C13}{C12}\right)\text{sample}/\left(\frac{C13}{C12}\right)\text{standard} - 1\right] \times 1000$$

The carbon isotope ratios ($^{13}C/^{12}C$) were expressed as relative values to the standard value of the Pee Dee Belemnite ($\delta^{13}C = 0.0112372‰$). The standards (acetanilide and spinach) were analyzed after every ten samples; the analytical precision of the instrument was ±0.13‰.

2.6. Statistics

To evaluate the differences in parameters among land use types, including soil enzymes, SOC, LOC, SC, C:N and the $\delta^{13}C$ values, we conducted one–way mixed–effect ANOVA (LSD test, $P = 0.05$) separately for each aggregate size, with land use as a fixed factor and block as a random factor. The normality and homoscedasticity of the residues were tested and verified, using the Kolmogorov-Smirnov test's and Levine's test, respectively. Similar tests were also carried out among different aggregate-sizes in the same land use type to analyze the impacts of aggregate-size on different parameters, with aggregate-size as a fixed factor and block as a random factor. The correlations between soil enzyme activities and soil chemical variables were based on the Pearson's correlation coefficients. All statistical analyses were conducted using SPSS 18.0 (SPSS, Chicago, IL, USA).

Structural equation modeling (SEM) was conducted to estimate the direct and indirect effects of aggregate-size on parameters related to SOC turnover according to the expectations of a priori model (Fig. S5; Shipley, 1999; Grace, 2006). SEM in afforested soils (shrubland and woodland) was carried out separately from soils that did not receive plant C inputs (open area and cropland). Soil aggregate composition and soil properties such as MBC and soil C:N ratios are major drivers of soil enzyme activities and SOC accumulation (Trasar-Cepeda et al., 2008; Dungait et al., 2012; Cheng et al., 2013; Cenini et al., 2016). SOC and soil enzyme activities are in reciprocal causation (Trasar-Cepeda et al., 2008; Burns et al., 2013; Cenini et al., 2016). On one hand, SOC

can significantly affect soil enzyme activities by providing energy and substrates for enzyme secretion (Allison et al., 2011; Cenini et al., 2016). In turn, enhanced soil enzyme activities can accelerate SOC decomposition, thus having negative impacts on SOC (Burns et al., 2013). Therefore, both the direct effect of SOC on soil enzyme activities and the vice versa were included in a priori model (Fig. S5). In addition, the direct effect of soil oxidase on soil hydrolases was incorporated into the model, as the increase in soil oxidase would reduce phenols that are toxic to hydrolases (Fig. S5; Freeman et al., 2001; Sinsabaugh, 2010).

The activities of four hydrolases were found to be highly positively correlated with each other (Table S2). Thus, a principal component analysis (PCA) was conducted to reduce the number of variables in the SEM. PCA was carried out by the principle component method (for factor extraction) using SPSS 18.0 (SPSS, Chicago, IL, USA). The principal component 1 (PC1) extracted from four hydrolases (BG, AG, CB and XYL) had an eigenvalue >1 and explained 74% of the total variance and was thus considered as the representative of the overall variation in hydrolases activities. Similarly, the first component of PPO and PO was also extracted as oxidase (eigenvalue >1) and explained 98% of the total variance. Both hydrolase and oxidase were introduced as new variables into the SEM. The distribution and normality of all endogenous variables were tested and verified. The normal-distribution-based maximum likelihood method was used for parameter estimations (Boldea and Magnus, 2009). The best-fitting model was selected by step-wise removal of non-significant paths ($P > 0.05$). The chi-squared test (χ^2; the model has a good fit when χ^2 is low and the P-value >0.05) and the root-mean-square error of approximation (RMSEA; the model has a good fit when $0 \le RMSEA \le 0.05$) were used to test the overall goodness of fit for the model (Schermelleh-Engel et al., 2003). All the SEM analyses were conducted using Amos 18.0 (IBM, SPSS, New York, USA).

3. Results

3.1. Soil aggregate size distribution, microbial biomass carbon and soil enzyme activity

Afforestation significantly promoted the percentage of macroaggregates (>2000 μm) in terms of total soil mass, accounting for 20.1%, 30.7%, 45.8% and 41.5% of total soil mass in the open area, cropland, shrubland and woodland, respectively (Table 1). MBC in bulk soil and all soil aggregate fractions were generally higher in the afforested soils compared to those in the open area and cropland (Fig. S1). MBC was lower in macroaggregates (>2000 μm) than that in soil mineral particles (<53 μm) across land use types (Fig. S1).

The BG, AG and XYL activities in bulk soil were significantly higher in the afforested soils than those in the open area and cropland (Fig. 1a, b, d). Soil AG and XYL activities in 250–2000 μm macroaggregates as well as soil BG and AG activities in 53–250 μm microaggregates were also significantly higher in the afforested soils compared to those in the open area and cropland. Soil hydrolase activities in <53 μm particles were generally the highest in the shrubland among all land use types. Soil hydrolase activities were generally lower in >2000 μm macroaggregates compared with those in other aggregate fractions following afforestation (Fig. 1). Land use type only had significantly effects on PPO and PO activities in >250 μm macroaggregates, with the highest PPO and PO activities seen in 250–2000 μm macroaggregates in the cropland (Fig. 2). Soil oxidase activities were generally highest in <53 μm soil mineral particles and lowest in 53–250 μm microaggregates across land use types (Fig. 2).

When normalized for SOC concentration in bulk soil and soil aggregate fractions (i.e., activity per unit SOC), specific hydrolase and oxidase activities in <53 μm mineral particles in the afforested soils were lower than those in the open area and cropland (Figs. S2 and S3). The specific hydrolase activities were generally lowest in >2000 μm macroaggregates compared with those in finer aggregate fractions across land use types, except that the lowest specific BG activity was observed in 53–250 μm microaggregates in the shrubland (Fig. S2).

Table 1
Aggregate size fraction distribution (%) under different land use types.

Land use	Aggregates (μm)	Aggregate fraction
		% of total soil mass
Open area	>2000	20.1 ± 2.9Cb
	250–2000	38.1 ± 3.3Aa
	53–250	25.2 ± 4.9Ab
	<53	13.4 ± 2.9Bc
	Recovery	96.8
Cropland	>2000	30.7 ± 2.2Bb
	250–2000	42.3 ± 6.5Aa
	53–250	9.8 ± 2.4Cc
	<53	14.5 ± 4.0ABc
	Recovery	97.3
Shrubland	>2000	45.8 ± 5.2Aa
	250–2000	26.7 ± 6.2Bb
	53–250	12.6 ± 1.0BCc
	<53	11.0 ± 0.7Bc
	Recovery	96.1
Woodland	>2000	41.5 ± 3.3Aa
	250–2000	25.2 ± 2.2Bb
	53–250	14.7 ± 1.8Bc
	<53	17.8 ± 1.7Ac
	Recovery	99.2

Note: MBC, microbial biomass carbon; capital letters indicate differences in the same aggregate among land use types; lowercase letters indicate differences in aggregate size fractions in the same land use type based on one-way mixed-effect ANOVA.

3.2. Soil C concentration, C:N ratio and the $\delta^{13}C$ value

Afforestation significantly increased SOC concentrations in bulk soil and all aggregate fractions compared to those in the open area and cropland (Fig. 3a). In particular, the woodland increased SOC concentration in >2000 μm macroaggregates by 4.2- and 3.2-fold, compared to the open area and cropland, respectively. SOC concentrations were significantly higher in >2000 μm macroaggregates compared with those in finer aggregate fractions across land use types (Fig. 3a). 51.2% and 55.7% of SOC was distributed in >2000 μm macroaggregates in the shrubland and woodland, respectively (Fig. S4b).

After treatment with NaOCl, the C concentration remaining in residues (SC) ranged from 0.5–2.2 g kg^{-1} (Fig. 3b). Across different land use types, NaOCl treatment removed 44–94% of SOC in bulk soil and aggregate fractions, with higher removal efficiency in the shrubland (84%–91%) and woodland (82%–94%) compared to those in the open area (44%–76%) and cropland (64%–81%). As a consequence, SC:SOC ratios were generally lower in the afforested soils than those in the open area and cropland (Fig. 3c).

The C:N ratios in bulk soil and all aggregate fractions were generally higher in the afforested soils (7 to 11 in shrubland, 5 to 14 in woodland) than those in the open area (5 to 9) and cropland (4 to 8), with the highest C:N ratio being observed in the woodland (Fig. 4a). Soil C:N ratios were generally lowest in <53 μm soil mineral particles and highest in >2000 μm macroaggregates across land use types. Treatment with NaOCl significantly reduced C:N ratios in all land use types (Fig. 4b).

The $\delta^{13}C$ values of soil organic pool in bulk soil and all aggregate fractions were significantly lower in the cropland, shrubland and woodland compared with those in the open area (Table 2). There were generally no significant changes in $\delta^{13}C$ values of soil organic pool among different aggregate fractions across land use types, with the exception of the significantly enriched $\delta^{13}C$ in 53–250 μm microaggregates in the open area and < 53 μm particles in the woodland. The $\delta^{13}C$ values of the stable (NaOCl–resistant) pool in bulk soil and all aggregate fractions

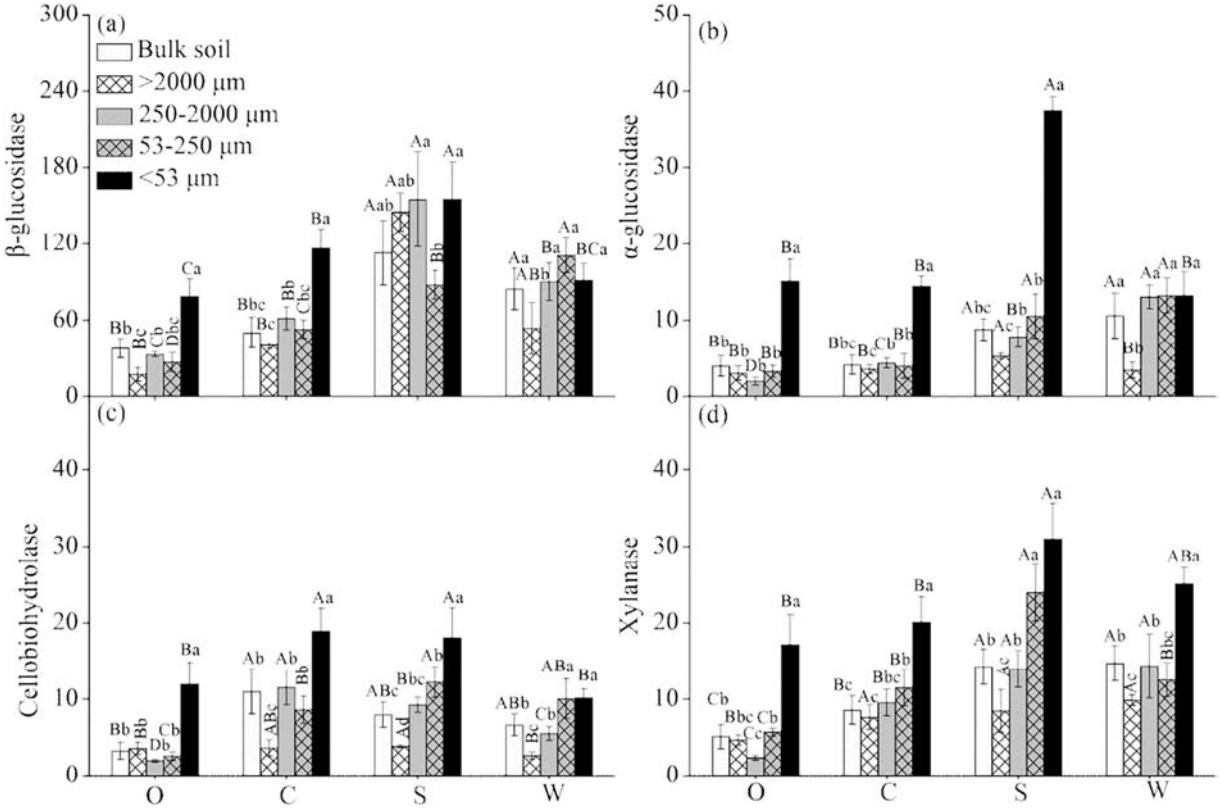

Fig. 1. Soil hydrolase activities in bulk soil and aggregates under different land use types in the Danjiangkou Reservoir area. Error bars represent standard deviation. O, Open area; C, Cropland; S, Shrubland; W, Woodland. Capital letters indicate differences in the same aggregate among land use types based on one-way mixed-effect ANOVA. Lowercase letters indicate differences in aggregate size fractions in the same land use type. Soil hydrolase activities were expressed as nmol product g^{-1} dry fraction h^{-1}.

were lower than those of soil organic pool in the cropland, shrubland and woodland (Table 2).

3.3. The relationships between soil enzyme activities and chemical variables related to C turnover

Soil hydrolase activities were negatively correlated with SOC and LOC in the afforested soils, whereas no significant correlation was found in the open area and cropland (Table 3). Soil BG, AG and CB activities were positively related with $\delta^{13}C$ values of soil organic pool in the cropland, while a positive correlation was observed only between AG activity and the $\delta^{13}C$ values in the shrubland. After the removal of LOC, both hydrolase and oxidase activities were positively correlated with the $\delta^{13}C$ values of stable pool in the afforested soils (Table 3). The $\delta^{13}C$ value of the organic pool was negatively correlated with SOC, LOC and soil C:N ratio in the afforested soils (Table S3).

3.4. The direct and indirect effects of aggregates on SOC turnover

Increasing aggregate size had directly positive effects on SOC storage related variables (SOC concentration and C:N ratio) but directly negative effect on soil hydrolase activities across land use types (Fig. 5). However, the indirect effects of increasing aggregate size on these SOC turnover variables were different among the land use types. In the open area and cropland, SOC concentration had a directly positive effect on hydrolase activities. In the shrubland and woodland, however, hydrolase activities had a negative effect on SOC concentration, and increasing aggregate size could indirectly increase SOC concentration via its negative effect on hydrolase activities.

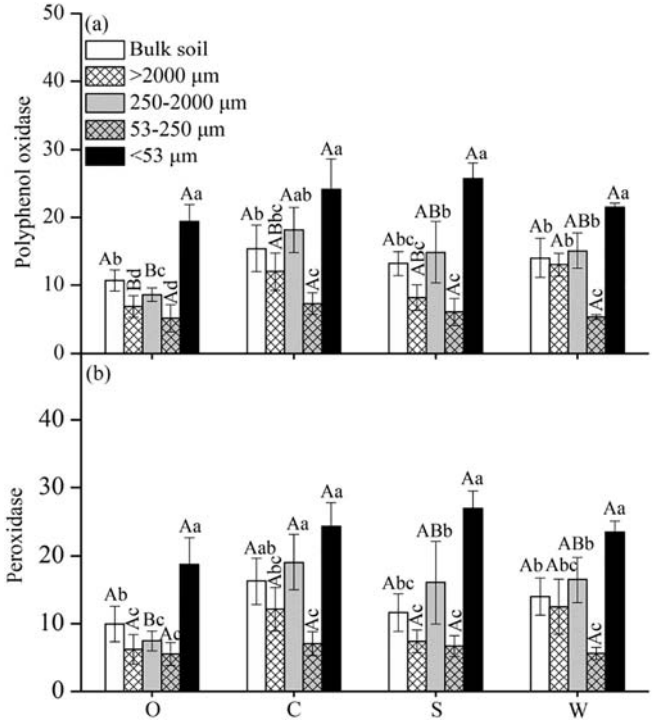

Fig. 2. Soil oxidase activities in bulk soil and aggregates under different land use types. Error bars represent standard deviation. See Fig. 1 for abbreviations and explanations of capital and lowercase letters. Soil oxidase activities were expressed as μmol product g^{-1} dry fraction h^{-1}.

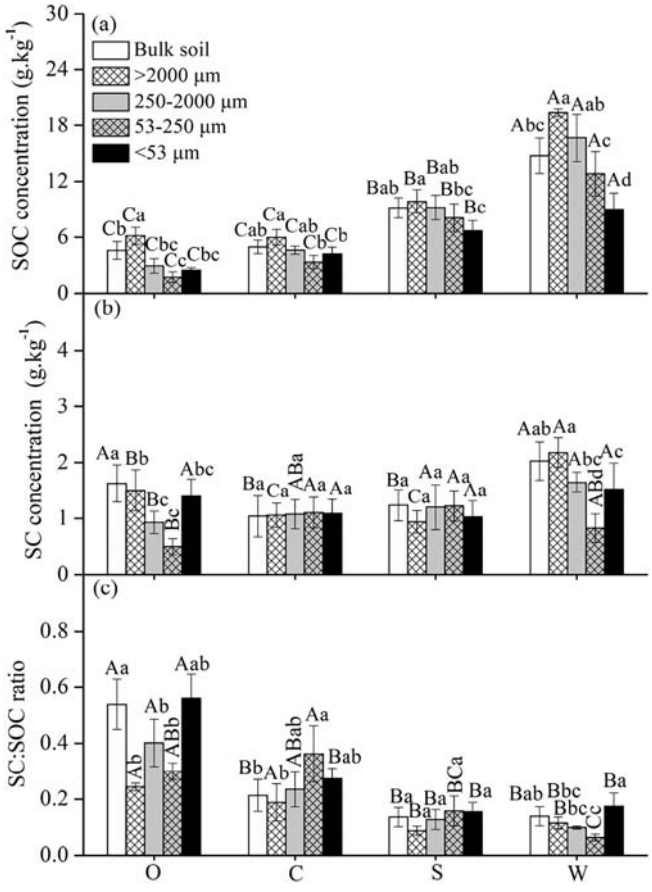

Fig. 3. Carbon concentration in total soil organic pool and stable (NaOCl-resistant) pool and their ratios in bulk soil and aggregates under different land use types. Error bars represent standard deviation. See Fig. 1 for explanations of capital and lowercase letters. SOC, soil organic C; SC, NaOCl-resistant stable C.

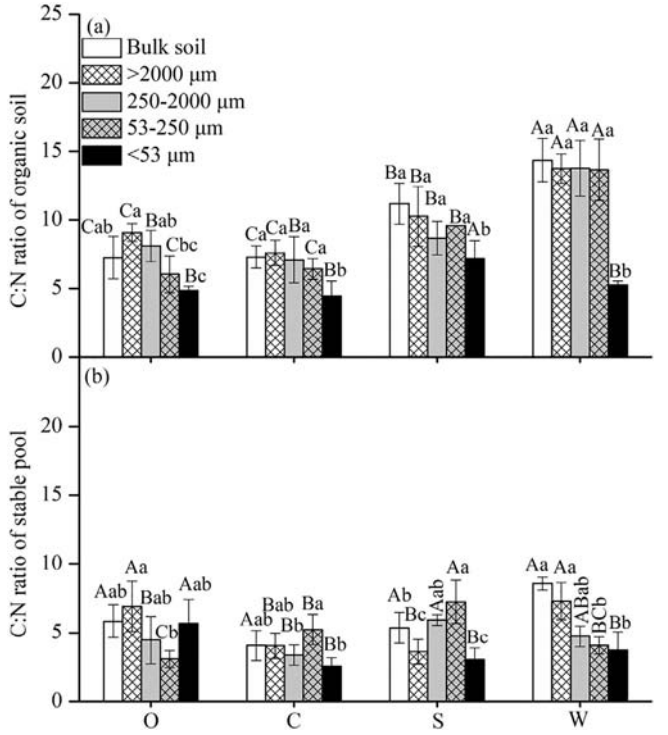

Fig. 4. The C:N ratios in total soil organic pool and stable (NaOCl-resistant) pool in bulk soil and aggregates. Error bars represent standard deviation. See Fig. 1 for explanations of capital and lowercase letters.

4. Discussion

4.1. Soil enzyme activities in bulk soil and soil aggregate fractions

Consistent with our hypothesis 1, soil hydrolase activities (including BG, AG and XYL) in bulk soil were higher in the afforested ecosystems compared with those in the open area and cropland (Fig. 1a, b, d). The higher soil hydrolase activities might be due to the higher MBC (Fig. S1) as well as higher labile SOC concentrations (Fig. 3) in the afforested soils than those in the open area and cropland, as soil enzymes came largely from microorganisms (Hammel et al., 2002; Tabatabai and Dick, 2002) and required adequate energy and substrates for enzyme production (Cenini et al., 2016). It was also notable that soil hydrolase activities in <53 μm particles in the shrubland were generally the highest among all land use types (Fig. 1). Similarly, the higher activities in <53 μm particles in the shrubland compared with those in the open area and cropland might be also associated with the generally higher labile SOC availability and higher MBC (Fig. 3; Fig. S1). However, the relatively higher activities in the shrubland than those in the woodland would be due to the relatively higher litter quality in the shrubland (Cheng et al., 2013). Compared with litters from leguminous plants in the shrubland, litters from coniferous plants in the woodland generally contained higher proportion of recalcitrant compounds such as lignin and aromatic C (Post and Kwon, 2000; Mendham et al., 2002; Wang et al., 2016), which would result in less labile SOC inputs into soil mineral particles and aggravate the limitation of labile C for enzyme production. In addition, the higher inputs of recalcitrant compounds in the woodland would also cause the denaturation of soil enzymes by synthesizing humic-enzyme polymers (Allison, 2006).

The PPO and PO activities of 250–2000 μm macroaggregates in the cropland were significantly higher compared to those in the afforested soils (Fig. 2), which was inconsistent with our hypothesis. It has been well established that there is selectively preservation of recalcitrant materials in the cropland (Cheng et al., 2013; O'Brien and Jastrow, 2013), which could be supported by the higher SC:SOC ratios in soil aggregate fractions in the cropland compared with those in the afforested soils (Fig. 3c). This selective preservation might induce the production of

Table 2
The $\delta^{13}C$ value (‰) in bulk soil and aggregates under different land use types.

Land use	Aggregates (μm)	$\delta^{13}C$	$\delta^{13}C$
		Soil organic pool	Stable pool
Open area	Bulk soil	−15.5 ± 0.4Ab	−13.3 ± 1.3Aa
	>2000	−15.6 ± 0.6Ab	−14.2 ± 2.8 Aa
	250–2000	−15.2 ± 0.5Ab	−14.7 ± 1.8Aa
	53–250	−14.9 ± 0.2Aa	−16.1 ± 0.6 Aa
	<53	−15.2 ± 0.1 Ab	−14.4 ± 1.8Aa
Cropland	Bulk soil	−24.5 ± 1.3Db	−27.2 ± 1.5Ca
	>2000	−23.9 ± 1.7Dab	−27.0 ± 0.4Ca
	250–2000	−23.5 ± 1.6Cab	−26.6 ± 1.6Ca
	53–250	−24.4 ± 0.5 Dab	−28.8 ± 1.1Ca
	<53	−22.9 ± 0.9Ca	−25.8 ± 1.5Ca
Shrubland	Bulk soil	−18.5 ± 0.4Ba	−20.1 ± 0.4Bb
	>2000	−17.9 ± 0.8 Ba	−19.7 ± 0.9 Bb
	250–2000	−18.6 ± 1.0ABa	−19.6 ± 0.5 Bb
	53–250	−18.6 ± 1.2 Ba	−20.7 ± 0.8 Bb
	<53	−18.3 ± 1.4ABa	−18.1 ± 1.3ABa
Woodland	Bulk soil	−19.7 ± 0.3Cb	−21.3 ± 0.3Bb
	>2000	−19.7 ± 0.5Cb	−21.0 ± 0.4Bb
	250–2000	−20.0 ± 0.3Bb	−21.1 ± 0.1Bb
	53–250	−20.3 ± 0.2Cbc	−21.0 ± 0.2 Bb
	<53	−19.2 ± 0.2Ba	−20.0 ± 0.2Ba

Note: Data shown are mean ± standard deviation. Capital letters indicate differences in the same aggregate among land use types based on one-way mixed-effect ANOVA. Lowercase letters indicate differences in aggregate size fractions in the same land use type. Soil organic pool represents the pool that washed with 0.5 M HCl to remove carbonates but untreated with NaOCl. Stable pool represents NaOCl-resistant pool.

Table 3
Correlations between soil enzyme activities and soil chemical properties in bulk soil and aggregates under different land use types.

Land use	Factors	SOC	LOC	SC	C:N	SC:N	$\delta^{13}C$	$S\delta^{13}C$
Open area	BG	−0.337	−0.307	0.147	−0.527*	0.016	0.070	−0.076
	AG	−0.260	−0.426	0.169	−0.565**	0.190	0.114	0.110
	CB	−0.130	−0.276	0.147	−0.482*	0.265	0.007	0.076
	XYL	−0.202	−0.344	0.107	−0.536*	0.170	0.095	−0.070
	PPO	−0.212	−0.375	0.378	−0.498*	0.291	0.015	0.146
	PO	−0.225	−0.394	0.368	−0.535*	0.247	−0.022	−0.003
Cropland	BG	−0.213	−0.229	0.114	−0.739**	−0.239	0.453*	0.401
	AG	−0.128	−0.126	0.003	−0.710**	−0.339	0.450*	0.482*
	CB	−0.293	−0.290	0.011	−0.644**	−0.229	0.417*	0.409
	XYL	−0.279	−0.286	0.063	−0.641**	−0.293	0.234	0.218
	PPO	−0.129	−0.080	−0.261	−0.333	−0.590**	0.264	0.504*
	PO	−0.084	−0.033	−0.274	−0.260	−0.581**	0.245	0.541*
Shrubland	BG	−0.426*	0.485*	0.202	−0.579*	−0.098	0.228	0.525*
	AG	−0.635**	−0.606**	−0.121	−0.922**	−0.496*	0.608**	0.860**
	CB	−0.604**	−0.615**	0.232	−0.740**	−0.488*	0.242	0.625**
	XYL	−0.551*	−0.621**	0.334	−0.631**	−0.486*	0.096	0.531*
	PPO	−0.491*	−0.485*	−0.022	−0.787**	0.328	0.310	0.653**
	PO	−0.471*	−0.474*	0.027	−0.803**	−0.357	0.363	0.664**
Woodland	BG	−0.431*	−0.459*	−0.612**	−0.476*	0.542*	−0.222	0.589*
	AG	−0.488*	−0.452*	−0.461*	−0.561*	0.356	−0.307	0.424*
	CB	−0.579**	−0.537*	−0.548*	−0.467*	0.282	−0.258	0.481*
	XYL	−0.613**	−0.640**	−0.071	−0.676**	−0.331	0.053	0.671**
	PPO	−0.298	−0.362	0.324	−0.310	0.714**	0.295	0.724**
	PO	−0.321	−0.385	0.315	−0.294	−0.651**	0.149	0.734**

Note: BG, β-glucosidase; AG, α-glucosidase; CB, Cellobiohydrolase; XYL, Xylanase; PPO, Polyphenol oxidase; PO, peroxidase; SOC, soil organic carbon. LOC, NaOCl-removable SOC; SC, NaOCl-resistant C; SC:N and $S\delta^{13}C$ represents C:N ratio and $\delta^{13}C$ value in stable (NaOCl-resistant) pool, respectively. *$P < 0.05$; **$P < 0.01$; numbers are correlation coefficients of Pearson correlation.

oxidases targeting these recalcitrant materials, facilitating the decomposition of relatively old SOC (Sinsabaugh, 2010). Indeed, our previous studies in the same study region have indicated that the decay rate of old C is higher in the cropland than that in the afforested soils (Cheng et al., 2013; Dou et al., 2016). In contrast, in the afforested soils where labile C from plant residues was abundant (Fig. 3; Hernandez-Ramirez et al., 2011; Wang et al., 2016), the decomposition of recalcitrant humic materials via oxidase was depressed, because the degradation of high resistance materials required high activation energy (Bauer et al., 2008).

The lower hydrolase activities (activity $soil^{-1}$) and specific hydrolase activities (activity SOC^{-1}) in >2000 μm macroaggregates compared with those in <53 μm soil mineral particles following afforestation supported our hypothesis 2 (Fig. 1; Fig. S2). These lower enzyme activities in macroaggregates were consistent with several previous studies (Stemmer et al., 1998; Fansler et al., 2005; Ling et al., 2014) and could be caused by a number of mechanisms. First, occlusion within aggregates could reduce the physical accessibility of soil organic compounds (substrate) to enzymes (Dungait et al., 2012; O'Brien and Jastrow, 2013). Second, inverse relationships of soil aggregate size with pores and the diffusion coefficient of oxygen have been observed by many studies (Elliott et al., 1980; Dungait et al., 2012; Zhang et al., 2015). Hence, oxidase activities in macroaggregates might be low due to the limitation of oxygen status (Freeman et al., 2004), which could further inhibit hydrolase activities because of phenols' toxicity to hydrolases (Freeman et al., 2001; Sinsabaugh, 2010). Another factor was that soil enzymes in macroaggregates would be limited by N availability in afforested soils (Smal and Olszewska, 2008; Sinsabaugh, 2010), as soil C:N ratio was higher in >250 μm macroaggregates compared with those in <53 μm soil mineral particles (Fig. 4a) and hydrolase activities were negatively correlated with C:N ratios in soil aggregate fractions following afforestation (Table 3). These variations in enzyme activities of different aggregate fractions suggest that soil enzymes and SOC decomposition is unevenly active within soil physical structure, which should be incorporated into conceptual models of enzyme functions and SOC turnover following afforestation (Dungait et al., 2012).

4.2. Linking enzyme activities to SOC turnover and sequestration

The variations in soil enzyme activities and SOC concentrations are intimately interlinked (Table 3; Trasar-Cepeda et al., 2008; Cenini et al., 2016). Our SEM showed that the effect of aggregate size on the relationship between SOC and soil enzyme activities was completely different in the afforested soils compared with that in the open area and cropland (Fig. 5). These paradoxical relationships were possibly ascribed to the complex relationships between SOC and soil enzyme activities (Trasar-Cepeda et al., 2008; Burns et al., 2013; Cenini et al., 2016). On one hand, the stimulation of enzyme activities could accelerate SOM decomposition rates, leading to SOC depletion (Burns et al., 2013). Therefore, the directly negative effect of hydrolase on SOC, coupling with the directly negative effect of soil aggregate size on soil hydrolase activities in the afforested soils (Fig. 5b), indicated that the promotion of macroaggregates under afforestation could indirectly promote SOC via reducing hydrolase activities. On the other hand, greater SOC accumulation could result in increased enzyme activities as a consequence of the wider range of substrates for decomposers (Cenini et al., 2016). Therefore, in the open area and cropland where new C inputs were scarce and SOC contents were low (Fig. 3a), decomposition via enzymes could be depressed due to the lack of energy and substrates (Allison et al., 2011; Cenini et al., 2016). These different linkages between SOC and soil enzyme activities across land use changes suggested that the enzyme activities were not simple reflections of SOC concentrations, changes in enzyme activities could actively affect SOC turnover and SOC sequestration in the afforested soils.

According to the isotopic fractionation hypothesis, organic compounds containing ^{12}C are usually more easily broken during enzyme-catalyzed decomposition than those containing ^{13}C (Garten et al., 2000; Peri et al., 2012). The decomposition of SOC via hydrolase activities in cropland thus led to the enrichment in soil ^{13}C, leading to positive correlations between $\delta^{13}C$ values and hydrolase activities in soil aggregates (Table 3). In the afforested soils, however, soil $\delta^{13}C$ values only showed a positive relationship with AG in the shrubland but exhibited significant negative correlations with SOC, NaOCl-removable LOC and C:N ratios (Table S3). One possible explanation was that the enrichment

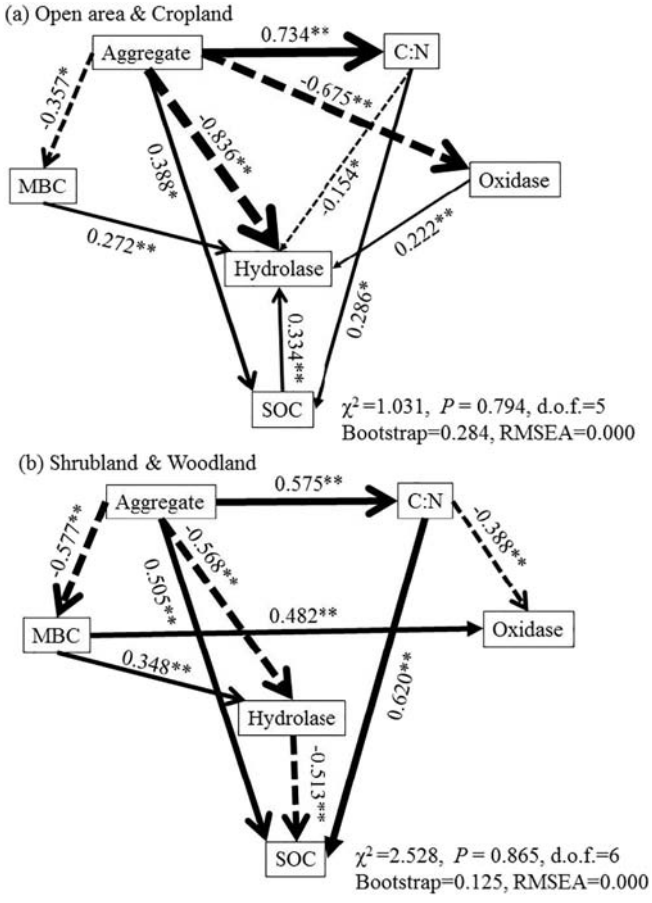

Fig. 5. Structural equation modeling assessing the direct and indirect effects of aggregate on soil organic carbon turnover under different land use types. Numbers adjacent to arrows indicate the effect size of the relationships; arrow thickness is proportional to the magnitude of path coefficient. Continuous and dashed arrows indicate positive and negative relationships, respectively. Significance levels are: $^{*}P < 0.05$; $^{**}P < 0.01$. Hydrolase was the first component of four hydrolytic enzyme activities (including β-glucosidase, α-glucosidase, cellobiohydrolase and xylanase) from principal components analysis; Oxidase was the first component of two oxidase enzyme activities (i.e., polyphenol oxidase and peroxidase). SOC, soil organic carbon; MBC, microbial biomass carbon.

of $\delta^{13}C$ as a result of enzyme-catalysis was offset by the increase in SOC derived from plant residues following afforestation, as plant-derived materials usually had lower $\delta^{13}C$ values (Garten et al., 2000; Cheng et al., 2013). These different relationships reflected a shift in the relative contributions of microbial decomposition vs. plant residues inputs to SOC dynamics across land use changes. After the removal of SOC that mostly derived from plant residues in the afforested soils, soil $\delta^{13}C$ values in the stable pool also correlated positively with soil hydrolases and oxidases activities (Table 3), confirming that soil enzymes were also important variables that contributed to C isotope composition and SOC dynamics following afforestation (Fansler et al., 2005; Guillaume et al., 2015). However, it is not always the case that higher soil enzyme activities will result in higher functions, because factors such as adsorption onto soil minerals may reduce the frequency of encounters between soil enzymes and SOC (Marx et al., 2005). Therefore, future investigations are needed to assess the kinetic characteristics of enzymes and their linkages to SOC and microorganisms, in order to confirm their functions in determining SOC sequestration.

4.3. Stabilization mechanisms on SOC sequestration

In the present study, it was observed that the NaOCl treatment removed 44–94% of SOC in the bulk soil and aggregate fractions across different land use types (Fig. 3). This finding was consistent with the reports of Zimmermann et al. (2007) and Mikutta et al. (2005). Organic compounds that were unprotected by crystalline Fe and Al oxides, even chemically complex SOC from newly input plant residues could mostly be removed during NaOCl extraction (Mikutta et al., 2006; Zimmermann et al., 2007). After the NaOCl treatment, $\delta^{13}C$ values in bulk soil and aggregate fractions declined in both the cropland and following afforestation (Table 2; Fig. 4), supporting previous studies that showed NaOCl treatments to preferentially remove ^{13}C-enriched C-containing compounds, leaving resistant materials depleted in ^{13}C (Helfrich et al., 2007; Zimmermann et al., 2007). Across different land use types, the low concentrations of SC indicated that the biochemical protection of SOC provided by high-specific surface area minerals was finite, consistent with recent evidence that SOC sequestration was more governed by physical accessibility than by biochemical recalcitrance (Dungait et al., 2012; O'Brien and Jastrow, 2013). Additionally, the lower SOC concentrations in the <53 μm soil mineral particles compared to those in macroaggregates (>250 μm) suggested that SOC protection through chemical binding was lower (Fig. 3; Six et al., 2002b; Dungait et al., 2012). These higher SOC concentrations in macroaggregates highlighted that physical protection in aggregates played essential role in SOC sequestration following afforestation. This finding provided an alternative pathway of SOC accumulation under afforestation, in addition to the widely observed pathway by increasing plant residues inputs (Post and Kwon, 2000; Cheng et al., 2013; Deng et al., 2016) and/or by increasing the contents of recalcitrant C fractions (Liu et al., 2017; Nath et al., 2018).

5. Conclusions

It is concluded that the distribution of soil C-hydrolyzing enzyme activities in the soil structure is essential component of SOC sequestration following afforestation in the Danjiangkou Reservoir area of central China. SOC sequestration in the afforested soils (shrubland and woodland) was intimately associated with a reduced percentage of SOC lost via enzyme decomposition in >2000 μm macroaggregates compared to finer soil aggregate fractions. Furthermore, our results from chemical fractionation after the physical separation of SOC pools confirmed that the physical protection of SOC in aggregates played essential role in SOC sequestration following afforestation. The combination of soil fractionation with enzymes and their interactions with SOC improved the detection of diverse relationships between SOC and soil enzyme activities. For example, a negative correlation between SOC and hydrolase was identified based on soil aggregates in the afforested soils, while the analysis of bulk soil was neither specific nor sensitive enough to detect this relationship. This negative relationship indicated that soil enzyme activities were not simple reflections of SOC, but actively affected SOC sequestration in the afforested soils, providing supplemented evidence for recent interests in how microorganism-mediated processes could cause soil C stabilization or loss (Zhang et al., 2015; Liang et al., 2017). Moreover, this could help reconcile the contrasting (i.e., positive, neutral and negative) responses of SOC sequestrations to afforestation in different regions (Smal and Olszewska, 2008; Mao and Zeng, 2010; Hernandez-Ramirez et al., 2011).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.05.332.

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